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Lethal and sublethal effects of the anti-sea lice formulation Salmosan® on the Pacific spot prawn (*Pandalus platyceros*)

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Abstract

The effects of the aquaculture chemotherapeutant Salmosan® (active ingredient [a.i.]: azamethiphos) were examined in Pacific spot prawns (*Pandalus platyceros*) at three temperatures (5, 11, and 17°C). Post-molt prawns were more sensitive to Salmosan® than intermolt prawns; repeated (3x) 1-hr LC50 values for post-molt prawns ranged from 17 (9.3–31 95% confident intervals) to 40 (25–63) μg/L a.i. while intermolt prawns survived 3 × 1-hr exposures up to 100 μg/L a.i. Using LC50 values, Salmosan® was approximately 2.4 times more toxic at 17 versus 5°C. Temperature significantly altered chemosensory and locomotory behaviors in intermolt prawns with the highest activity at the intermediate temperature. Significant decreases in antennule flicking (84 and 104% over controls) were seen at 17°C after 3 × 1-hr pulse exposures to 50 and 100 μg/L a.i., respectively. Temperature, but not Salmosan®, affected molting success: at 17°C significantly lower survival was seen during ecdysis (60% of those at 5°C) and at 5°C, molt time was longer (41 ± 3 days) compared to 11°C (34 ± 4 days) or 17°C (21 ± 4 days). Life stage (molt status) and environmental parameters (temperature) alter the effects of Salmosan® to non-target spot prawns.

KEYWORDS
aquaculture, azamethiphos, behavior, chemotherapeutant, molt, pesticide, sea lice, toxicity

1 | INTRODUCTION

Declining fish stocks worldwide have prompted a rapid expansion of the aquaculture industry to meet the growing global demand for seafood. Aquaculture is estimated to make up 46% of the world's seafood production by tonnage and 62% of the total sale value (FAO, 2020). In Canada, aquaculture accounts for roughly 20% of total seafood production. This industry generates $1.4 billion in revenue annually and creates 14,000 full time equivalent jobs (DFO, 2020). The largest contributor to Canadian aquaculture in both tonnages harvested and economic value is the open net pen industry, accounting for just over half of all aquaculture production (Statistics Canada, 2018).

Open net pens in near-shore coastal waters benefit from the free flow of seawater and natural fluctuating water conditions including salinity, temperature, and dissolved oxygen. Open net pen immersion in the marine environment may pose risks to proximal ecosystems through the release of biological and chemical pollutants. Of particular importance is the facilitated proliferation of pathogens and parasites in densely populated net pens and the resulting pest management strategies employed. For example, outbreaks of ecto-parasitic sea lice have implications for both farmed and wild salmon health, and chemical treatment options pose risks to non-target organisms (Costello, 2009). Information on chemotherapeutant chemicals and appropriate sea lice management programs is essential to industry success and protecting ecosystem health.

In Canada, there are three chemotherapeutant formulations currently approved by Health Canada for treatment against sea lice outbreaks: one in-feed treatment, SLICE®; and two water-soluble bath treatments, Paramove®50 and Salmosan® (PMRA, 2016). On the Pacific coast of Canada, SLICE® remains the most common anti-sea lice chemotherapeutant in use. However, concern regarding the reliance on SLICE® and the potential for resistance to develop in sea lice was the impetus for the full registration of Paramove®50 and Salmosan® for use in salmonid aquaculture on the west coast of Canada (PMRA, 2016).

Bath treatments of Salmosan® occur either directly in net pens or fish are transported into and treated in specialized well-boats. Following bath treatment, contaminated water is released directly into the surrounding water and diluted, creating the potential for short-term, low concentration exposures to non-target organisms. Salmosan® treatments are often applied consecutively to target all life stages of sea lice, to treat multiple pens within one farm, or to treat multiple farms within a single area, resulting in non-target organisms being subjected to repeated pulses of this formulation (Burridge, Haya, & Waddy, 2008; Dounia, Andrea, Lefort, & Van Geest, 2016).

The active ingredient (a.i.) in Salmosan®, the organophosphate azamethiphos, is an inhibitor of acetylcholinesterase (AChE), which terminates impulse transmission in cholinergic pathways in nervous systems by the rapid hydrolysis of the neurotransmitter acetylcholine (ACh). The accumulation of ACh in the synapse results in repeated post-synaptic action potentials and insensitivity to further signaling. AChE inhibition at the whole animal level results in convulsions, twitching, agitation, and eventual partial or complete paralysis (Couillard & Burridge, 2014) and death by hypoxia through the paralysis of respiratory muscles (Dounia et al., 2016). Paralysis may indirectly lead to reduced survival or fitness through reduced performances that rely on a properly functioning nervous system (e.g., an inability to forage or escape from predators). ACh is the primary sensory neurotransmitter in many crustaceans (Florey, 1973), therefore AChE inhibition may also interrupt responses to environmental chemical cues and signals, having downstream consequences on signal-reliant activities (e.g., foraging, mating, detecting predators).

The Pacific spot prawn *(Pandalus platyceros)* is an important non-target species as its habitat overlaps with areas occupied by open net pen salmon farm operations and has both economic and cultural importance. Prawns congregate near net pens, being attracted to fecal matter and detritus falling from pens, as has been reported for American

lobsters in Atlantic Canada (Findlay, Watling, & Mayer, 1995). The near-shore environment where aquaculture exists also provides migration corridors from deep waters to shallow intertidal areas where nocturnal foraging occurs (Butler, 1964). Increases in the numbers of dead shrimp and crabs near aquaculture sites following well-boat treatments using Salmosan® have been reported in Atlantic Canada (Wiber, Young, & Wilson, 2012) and it has been reported that azamethiphos exposure can adversely affect some species of non-target crustaceans (Burridge, Haya, & Waddy, 2005).

Limited data exist on the effects of Salmosan® on this Pacific prawn species, a likely representative species for prawns that will be exposed near aquaculture operations; therefore information on the lethal and sublethal effects from short-term, low concentration pulse exposures of Salmosan® that would simulate repeated releases that result from treating sea lice outbreaks (Burridge, Haya, Waddy, & Wade, 2000; Dounia et al., 2016; Van Geest, Burridge, Fife, & Kidd, 2014) are essential. Prawns can experience extremes in water temperature; the literature indicates that ambient temperature change can modify the response of ectothermic organisms to chemical exposures (Kennedy & Walsh, 1997). Life history stage susceptibility is also of practical importance in determining crustacean risk to chemotherapeutant exposures. The hypothesis being tested in the present study is that Salmosan® exposure affects the lethality, chemosensory, and locomotory behaviors and molting in spot prawn and these effects are modulated by environmental temperature.

2 | METHODS

2.1 | Prawns

Adult Pacific spot prawns (*Pandalus platyceros*) were purchased from a local vendor and transported in coolers containing source seawater at approximately 10°C under constant aeration. Prawns were held communally in 500 L holding tanks containing filtered and sterilized natural seawater (source: Vancouver Aquarium [Vancouver, BC], treated by slow sand filtration and disinfected with ultraviolet [UV] radiation) at 11 ± 1°C under constant aeration, with a pH range of 7.5–8.5, dissolved oxygen between 90 and 100% saturation (approximately 7.5–8.5 mg/L), salinity between 28 and 32, and a 12:12 photoperiod. The maximum loading density was 1 prawn/5 L seawater. Prawns were fed a mixed diet of frozen brine shrimp, fish, and squid ad libitum 3 to 4 times per week. Prawns were monitored daily for molting during a molting period. One hour after feeding, any remaining food and feces as well as 50–60% of the water were siphoned from holding tanks and replaced with fresh seawater. All prawns were acclimated for at least 2 weeks prior to an experiment.

Intermolt prawns were defined as prawns that were not preparing to undergo and fully recovered from a recent molting event (Chang, 1995; Hosamani, Reddy, & S. and Reddy P, R., 2017). Pre-molt prawns are not easily identified, therefore, to ensure that prawns were in the intermolt stage during exposure, prawns that molted within 1 week of exposure were removed from further analyses. Prawns were defined to be in the post-molt stage if they had molted within the previous 24 hr period. Therefore, in order to expose post-molt prawns, intermolt prawns were held individually within 9 L glass aquaria in water baths held at 5, 11, or 17°C. Prawns were checked for molting each day at 8:00 a.m. and, if molting occurred, exposed by 9:00 a.m. Temperature, dissolved oxygen, pH, and conductivity were recorded during exposures and three times a week during the monitoring period.

2.2 | Chemicals

Salmosan® (Fish Vet Group®, Inverness, Scotland) (wettable powder formulation) containing 49.8% a.i. azamethiphos was obtained from Fisheries and Oceans Canada (DFO). Animals were euthanized with 1 g/L ethyl 3 amino benzoate methanesulfonate (MS222; Sigma Aldrich, ON) buffered with 1 g/L sodium bicarbonate (Sigma Aldrich, ON). A

solution of 1 M L-glutamate (Sigma Aldrich, ON, Canada) in seawater was prepared as a surrogate food stimulant for behavioral experiments.

Water samples (1 L) from representative exposure tanks were collected in amber vessels and preserved with 2 g NaCl and 5 mL chloroform, shaken and then stored at 4°C until analysis for the active ingredient azamethiphos (Burridge, Haya, Zitko, & Waddy, 1999) and analyzed according to Strachan and Kennedy (2021). Briefly, samples were extracted using 2 g NaCl/100 mL water (Burridge et al., 1999), followed by adding 50 mL dichloromethane (DCM) (Van Geest et al., 2014). Dried extracts were combined and evaporated under N_2 and then reconstituted in methanol, eluted through graphitized carbon and alumina SPE cartridges with DCM, and then dried. The residue was taken up in HPLC-grade acetonitrile and analyzed by HPLC on a Hewlett Packard Model 1050 with an HP Model 1046A programmable fluorescence detector (FLD) and HP 3396 Series II Integrator (Hewlett-Packard) (Strachan & Kennedy, 2021). The detection limit for azamethiphos using 1-L water samples was 1.5 μg/L and the calibration curve linear for the test concentration range.

2.3 | Exposures

Prawns were acclimated slowly to an exposure temperature (5, 11, or 17°C) over 24 hr before an exposure (Tu et al., 2012). In several experiments (Sections 2.4, 2.5, and 2.6), individual prawns were subjected to short-term (1 hr) pulse (3x) Salmosan® exposures (0, 10, 50, or 100 μg/L a.i. azamethiphos) at three temperatures in 9 L glass aquaria. Pulse exposures were conducted at 0, 6, and 24 hr for acute lethality tests (Section 2.4) and molting experiments (Section 2.6) and at 0, 2.5, and 5 hr for behavioral experiments (Section 2.5). Exposure regimes were based on preliminary experiments and are environmentally relevant. Prawns were held in uncontaminated water between pulse exposures. Target azamethiphos concentrations were achieved by adding appropriate volumes of a thoroughly mixed (1 hr) Salmosan® in seawater solution to aquaria. Chemical analysis for the a.i. azamethiphos was performed on representative water samples (range 1–100 μg/L) in experiments (methods fully described in Strachan & Kennedy, 2021); analysis resulted in measured concentrations being 95 ± 4% of all target concentrations. Water temperature, dissolved oxygen, pH, and conductivity were recorded during exposures.

2.4 | Acute lethality in intermolt and post-molt prawns

Intermolt and post-molt prawns were subjected to 3 × 1-hr exposures to Salmosan® at $t = 0$, 6, and 24 hr at water temperatures of 5, 11, or 17°C (Section 2.3). For each temperature, 10 intermolt prawns were used per treatment concentration. Post-molt prawns were less readily available and at 5 and 11°C, six post-molt prawns were subjected per treatment concentration. Pre-exposure mid-ecdysis mortalities at 17°C resulted in $n = 4$ to 6 post-molt prawns subjected to each treatment concentration at this temperature. Due to the short duration of exposures, following the final pulse exposure, prawns were transferred to clean water and monitored for 24 hr for survival.

2.5 | Effects on chemosensory-mediated behaviors

Intermolt prawns were subjected to 3 × 1-hr exposures to Salmosan® at $t = 0$, 2.5, and 5 hr at 5, 11, or 17°C (Section 2.3; $n = 6$ prawns per treatment concentration). Each prawn was then individually transferred to a clean aquarium and subjected to a behavioral test at 24 and 96 hr following the first exposure. Behavioral tests were performed in blacked-out 9 L glass aquaria maintained at exposure temperatures. During the behavioral tests, a prawn's

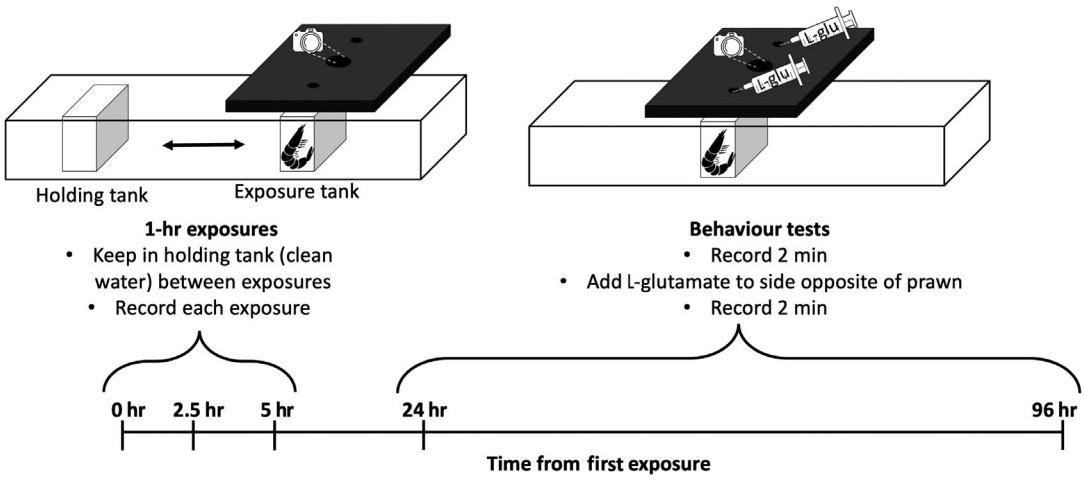

FIGURE 1 Schematic diagram of the behavior experiment. Prawns were held individually at 5, 11, or 17°C and subjected to 3 × 1-hr exposures to 0, 10, 50, or 100 μg/L azamethiphos in formulation as Salmosan®. Behavior tests were conducted at 24 and 96 hr post-exposure, during which the response to a food stimulant, L-glutamate (L-glu), was video-recorded

TABLE 1 Definitions for prawn behaviors examined in behavioral experiments (adapted from Lee and Meyers [1996] and Park [2013])

Behavior	Definition
Movement	Action of pleopods or pereiopods and a gain of distance. Includes swimming and walking
Antennule flick	Short, quick, complete jerk of antennule. Does not include slow, complete jerk
Antennule wipe	Sweep, or brush, of antennule through maxillipeds
Antennae wipe	Sweep, or brush, of antennae through maxillipeds
Dactyl probe	Complete extension and contraction of dactyl. Includes long and short probing events
Reaction	Dactyl probe or initiation of movement

responses to a surrogate food stimulant (L-glutamate) were measured (Atema & Stein, 1974). Prawns were acclimated (30 min) to the behavioral test tanks with a plexiglass cover that allowed for video recording and the addition of an aliquot (2 mL) of a 1 M L-glutamate solution (Figure 1). Prawns were recorded for 2 min (time frame for unexposed prawns to respond) to assess baseline behavior in the absence of L-glutamate. Following baseline recording, the L-glutamate solution was introduced into the end of the tank opposite to the side the prawn resided on, and video recorded for another 2 min to record responses. At the termination of the test, prawns were transferred into a separate 9 L aquarium containing clean seawater at the same temperature. The test was repeated again at 96 hr. Temperature, dissolved oxygen, pH, and salinity were measured before each behavioral test.

Video recordings were analyzed for a suite of common prawn behaviors that included total time spent moving (walking or swimming), number of antennule flicks, antennule wipes, antennae wipes, and dactyl probes during the 2-min periods before and after L-glutamate addition (Table 1). The latency time to react (i.e., dactyl probe, walk, or swim) after L-glutamate addition was also determined from recordings. Therefore, the response to L-glutamate was represented by five separate metrics (Table 1).

2.6 | Effects on molt-related endpoints

Intermolt stage prawns were subjected to 3 × 1-hr exposures to Salmosan® at $t = 0$, 6, and 24 hr at 5, 11, or 17°C (Section 2.3; $n = 10$ prawns per treatment concentration). Prawns were then transferred into uncontaminated water in 9 L tanks and monitored daily for molting. During the monitoring period, prawns were fed as described in Section 2.1. Prawns were euthanized 7 days after molting.

Molting success (defined as survival for 7 days following ecdysis) was recorded as well as the time to molt (day between the final pulse exposure and the initiation of ecdysis). Carapace length (CL) measurements were recorded at time of ecdysis (by measuring the CL of the shed exuvia [CL_{exuvia}]) and 7 days following ecdysis (CL_{7d}). Carapace length was measured 7 days after molting as growth occurs during this period through the continuous intake of seawater, increasing body volume and expanding the new, malleable exoskeleton (Chang, 1995; Hosamani et al., 2017). Relative CL growth was then calculated for each prawn as (Prentice & Rensel, 1977) using: Relative CL growth $= (CL_{7d} - CL_{exuvia})/CL_{exuvia}$ X 100%.

Sex was determined at the time of euthanization (by the presence of the appendix masculina [male] on the second pleopod (Butler, 1964). Mass was measured 1 day following exposure as well as at the time of euthanization ($mass_{7d}$). Prawns have typically ceased seawater intake by 7 days following ecdysis and it was expected that $mass_{7d}$ represented a stable measurement (Chang, 1995; Hosamani et al., 2017). Fulton's K condition factor was then calculated for each prawn (Gopalakrishan et al., 2014) using: Fulton's K condition factor $= mass_{7d}/CL_{7d}^3 \times 100$.

2.7 | Statistical analysis

LC50 values and their 95% confidence intervals for the post-molt and intermolt 3 × 1-hr exposures at each temperature were estimated using the Spearman Karber method (ecotoxicology package) in R (Gama, 2015; R Development Core Team, 2017). LC50 values were compared between each temperature treatment group using the ratio test (Wheeler, Park, & Bailer, 2006). For the exposures in which no LC50 could be determined, the highest concentration without a significant difference in mortality compared to the control groups was reported, representing the no observed effects concentration (NOEC).

To determine if exposure resulted in behavioral alterations, responses (numbers of flicks, wipes, probes, and time spent moving) before and after L-glutamate addition were compared between treatments using a two-way analyses of variance (ANOVA) followed by a Tukey's post hoc test. Temperature, azamethiphos concentration, and the interaction term were included as fixed effects. These analyses were run in JMP® version 13.1.0 (SAS Institute Inc., 2016). Data are presented as mean ± standard error. Since not all prawns displayed a reaction to L-glutamate, a Kaplan–Meier curve was created for each treatment group for the mean latency to respond (Rich et al., 2010). In this approach, each prawn was provided a status as "reacted" or "did not react," and a time-to-event (i.e., time to react, or time at last observation). The Kaplan–Meier curves for each treatment were compared using a log-rank test with a Benjamini-Hochberg adjusted p-value to account for multiple comparisons. These analyses were run in R version 1.1.4 using the survminer package (Kassambara & Kosinski, 2018; R Development Core Team, 2017).

A generalized linear mixed effects model (GLMM) was used to determine the effects of temperature and concentration on survival during molt using the lme4 package in R version 1.1.4 (Bates, Maechler, Bolker, & Walker, 2015; R Development Core Team, 2017). Separate two-way ANOVAs were used to determine the effects of temperature and concentration on time to molt, relative CL change, relative change in mass and Fulton's K in JMP® Version 13.1.0 (SAS Institute Inc., 2016). All analyses included water baths as random effects. A p-value of $<.05$ was used to infer statistical significance.

TABLE 2 Estimated LC50 values (95% confidence intervals) and NOECs for intermolt and post-molt prawns subjected to 3 × 1-hr exposures of Salmosan® (active ingredient: azamethiphos) at $t = 0, 6, 24$ hr

			Pulse 3 LC50	Pulse 3 NOEC
Life stage	Temperature (°C)	n	µg/L azamethiphos (95% CI)	µg/L azamethiphos
Intermolt	5	40	>100	>100
Intermolt	11	40	>100	>100
Intermolt	17	40	>100	>100
Post-molt	5	24	39.8[a] (25.1–63.0)	10
Post-molt	11	24	27.1[ab] (19.1–38.2)	10
Post-molt	17	20	17.1[b] (9.3–31.4)	10

Note: Identical superscript letters indicate no significant difference in LC50 values between temperature treatment groups in post-molt prawns (alpha = .05).

3 | RESULTS

3.1 | Water quality and chemistry

Water temperature during exposures, monitoring periods, and behavior tests remained within ±1.5°C of all target temperatures. Dissolved oxygen ranged from 7.8 to 9.1 mg/L, pH ranged from 7.8 to 8.7, and conductivity ranged from 42.2 to 52.3 µS/cm^2. Values of measured azamethiphos concentrations in exposure tanks from representative samples were typically >92 ± 5% of nominal concentrations, therefore the results are expressed in terms of nominal values.

3.2 | Acute lethality

Intermolt prawns survived exposure to Salmosan® at all concentrations tested (NOEC value 100 µg/L a.i.) for all three temperatures (Table 2). Salmosan® exposure caused mortality in post-molt prawns in both a concentration- and temperature-dependent manner (Table 2). The 3 × 1-hr LC50 (95% confidence interval) values were 40 µg/L (25–63 µg/L), 27 µg/L (19–38 µg/L), and 17 µg/L (9.3–31 µg/L) a.i. azamethiphos at 5, 11, and 17°C, respectively. The LC50 value at 17°C was significantly lower than the value determined at 5°C ($p = .045$). The 11°C LC50 value was not statistically distinguishable from the values at 5 or 17°C ($p > .05$).

3.3 | Effects on chemosensory-related behaviors

Temperature alone affected two measured behaviors in spot prawns: antennule flicking and locomotory behavior. In prawns held at 11°C, antennule flicking and time spent in locomotion (walking or swimming) in response to L-glutamate exposure were greater when compared to prawns held at either 5 or 17°C ($p < .05$; Figure 2). Salmosan® affected behavioral responses to L-glutamate in prawns held at 17°C but not those held at 5 or 11°C ($p > .05$; Figures 2 and 3). Prawns held at 17°C and exposed to 50 and 100 µg/L a.i. azamethiphos showed an 84 and 104% reduction in antennule flicking, respectively, 24 h following exposure compared to the same temperature controls ($p < .05$; Figure 2) with no significant effects in other behaviors. No effects on any behavior in any treatment group were seen 96 hr following exposure ($p > .05$). Salmosan® did not affect other behaviors at 17°C ($p > .05$; Figures 2 and 3).

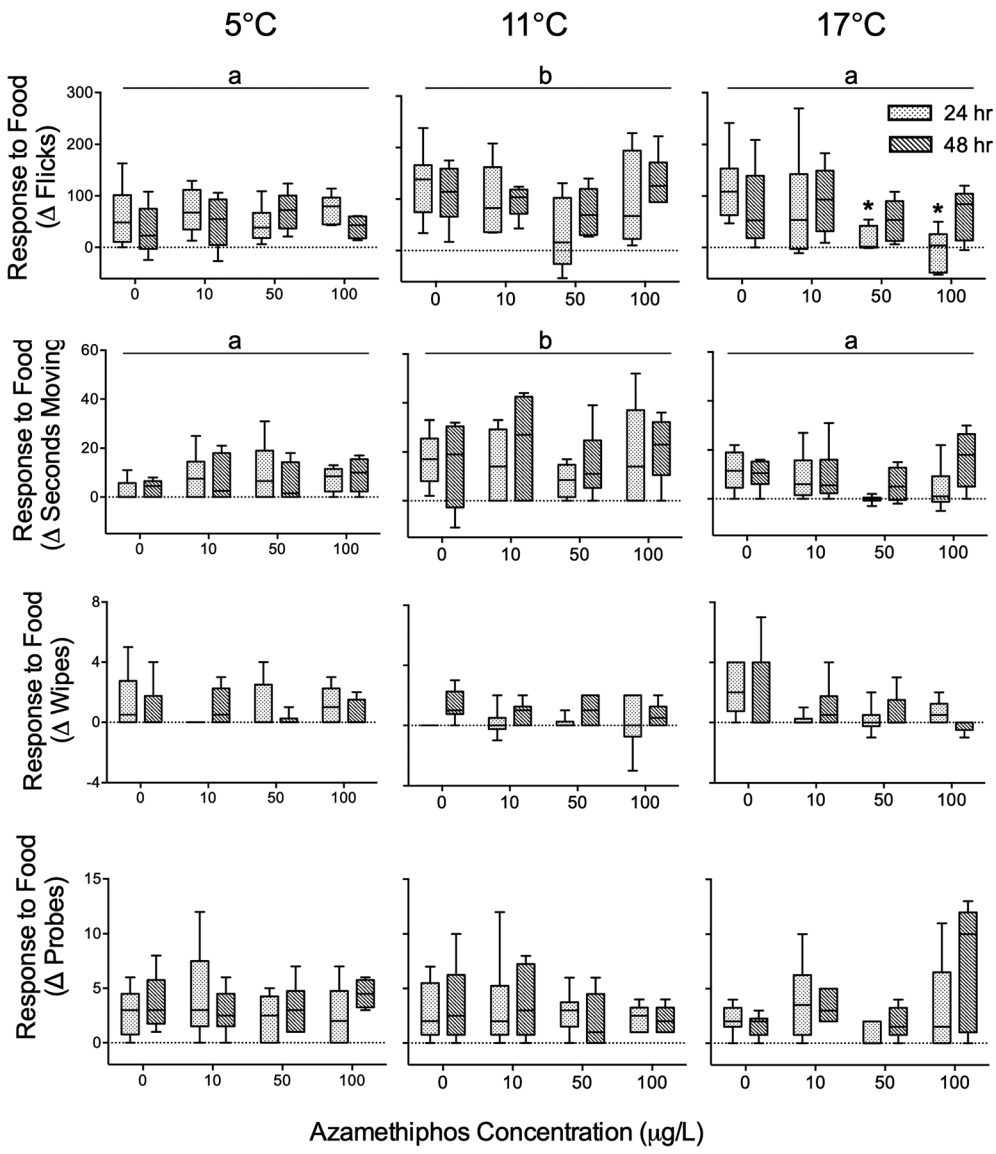

FIGURE 2 Feeding behaviors in spot prawns measured in response to L-glutamate at 24 and 96 hr following 3 × 1-hr exposures ($t = 0$, 2.5, 5 h) to Salmosan® (active ingredient: azamethiphos) at three temperatures. Values were determined as the Δ in a behavior = number before stimulus − number of activity following stimulus. There was evidence of an effect of Salmosan® exposure on antennule flicking at 24 hr but not 96 hr in prawns exposed to 50 and 100 μg/L at 17°C (top panel; $p < .05$). Prawns held at 11°C moved more than at 5 or 17°C at both 24 and 96 hr (second panel; $p < .05$). Lines indicate the medians; boxes indicate the first and third quartiles; whiskers indicate the minima and maxima. Asterisks indicate a significant difference ($p < .05$) from the control group at the same time point

3.4 | Effects on molt-related parameters

Temperature alone affected molting success: significantly lower survival (all mortalities occurred during ecdysis) was noted following molting in control prawns held at 17°C (60%) compared to 5 (100%; $p = .014$) or 11°C (90%;

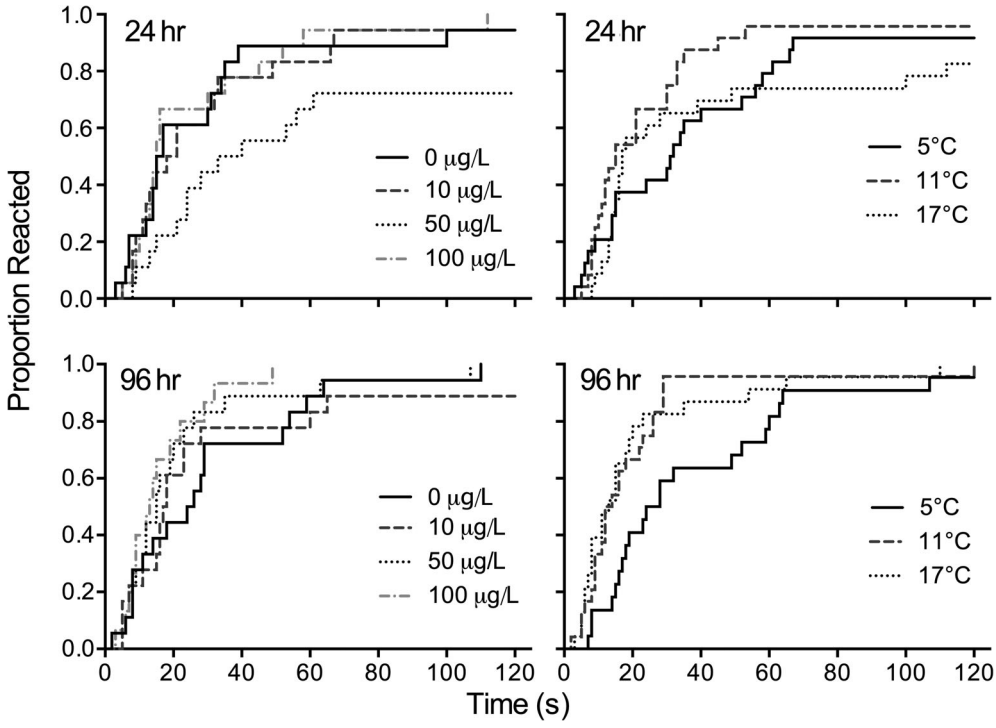

FIGURE 3 Kaplan–Meier curves showing the proportion of prawns that reacted to L-glutamate, a food stimulant, over time at $t = 24$ and 96 hr after 3 × 1-hr exposures ($t = 0$, 2.5, 5 h) to Salmosan® (active ingredient: azamethiphos) at three temperatures. A response was defined as either dactyl probing or initiation of movement. There was no effect of Salmosan® or temperature on time to react at 24 or 96 hr ($p > .05$)

$p = .008$; Figure 4). Temperature also affected the meantime to molt, with control prawns held at 5°C taking longer to molt (41 ± 3 days) compared to those at 11°C (34 ± 4 days) or 17°C (21 ± 4 days; $p < .05$; Figure 4). There was no effect of temperature in control prawns in relative CL growth ($p > .05$; Figure 4), relative change in mass ($p > .05$; Figure 4), or condition factor ($p > .05$; Figure 4). Salmosan® did not affect molting success, time to molt, relative CL or mass growth, or condition factor at any temperature ($p > 0.05$, Figure 4).

4 | DISCUSSION AND CONCLUSIONS

Pacific spot prawns were exposed to azamethiphos in formulation as Salmosan® at several environmental temperatures within their natural tolerable range and assessed for lethal and sublethal toxicological effects that relate to chemosensation and molting. The environmental temperatures used did not affect the survival of intermolt and postmolt spot prawns but did significantly alter measured chemosensory behaviors and molting.

Prawns expressed depressed antennule flicking and locomotion at 5 and 17°C compared to 11°C. Spot prawns can experience 17°C water temperatures for short periods of time during nocturnal foraging in surface waters but spend the majority of their lives in the benthic zone where temperatures typically range from 6 to 12°C (Butler, 1964). The spot prawn zone of resistance for temperature likely includes 17°C; however, prawns may be unable to fully acclimate and only survive for a limited period of time at this temperature. Temperature acclimation periods for crustaceans in previous studies range from 24 hr (e.g., Tu et al., 2012) to 30 days (e.g., Manush, Pal, Chatterjee, Das, & Mukherjee, 2004). Prawns were acclimated slowly to 17°C for 24 hr in these experiments and a longer

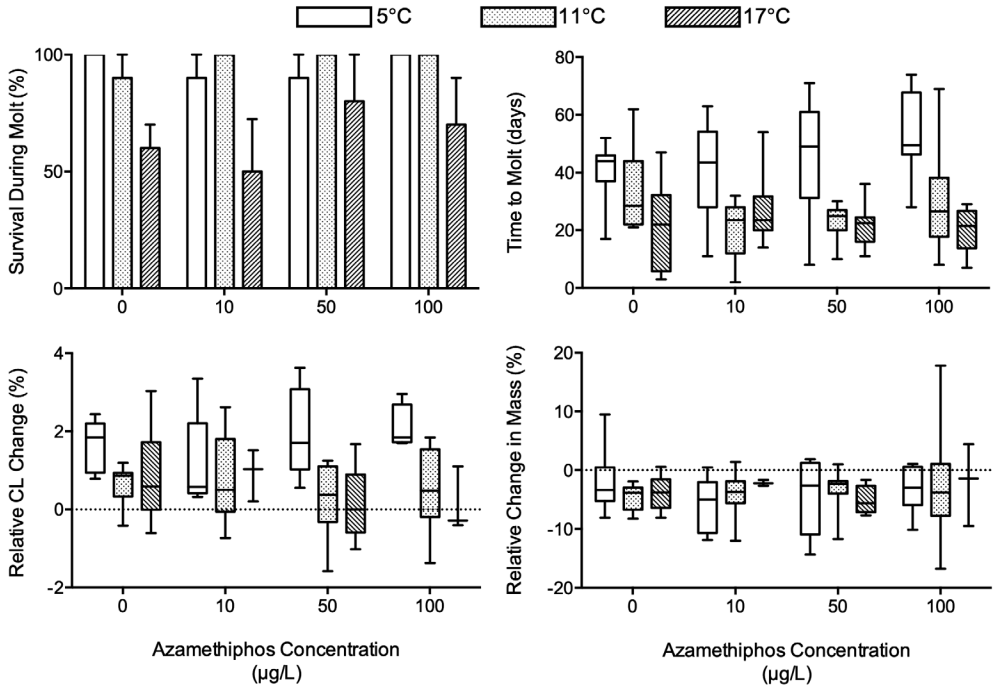

FIGURE 4 Effect of Salmosan® and temperature on survival during molt (top left), time to molt (top right), change in carapace length (bottom left), and change in mass (bottom right) of Pacific spot prawns after 3 × 1-hr ($t = 0, 6, 24$) exposures to Salmosan® (active ingredient: azamethiphos) at various temperatures. There was no effect of Salmosan® on any molting endpoint ($p > .05$). Prawns at 17°C had lower survival than at 5°C ($p = .014$), or 11°C ($p = .008$). Prawns held at 5°C took longer to molt than at 11°C and 17°C ($p < .05$). Bars indicate mean values; whiskers indicate standard error

time frame may be needed for full acclimation. The short acclimation period used was intentional since prawns typically move between waters of very different temperatures rapidly and are considered eurythermal and are not likely to fully acclimate to novel temperatures in their natural environment when exposed (Butler, 1964).

Prawns experienced faster molting and lower molting success with higher temperature. Temperature increases metabolism in ectothermic organisms and may underlie the increase in the rate of crustacean molting (Arnberg et al., 2013). The molting cycle is partially regulated by external cues, and increased temperature can act as a cue to initiate molting (Chang, 1995). Inappropriately timed cues may have resulted in higher rates of mortality during ecdysis as accelerated molting can limit physiological preparation for ecdysis (Chang, 1995). The results here are consistent with previous studies; for example, larval spot prawn reared at >21°C experience higher rates of mortality during ecdysis than those reared between 9.5 and 21°C (Kelly, Hastline, & Ebert, 1977). As well, *Pandalus borealis* embryos hatch earlier and pass through successive larval stages faster at elevated temperatures (9.5 vs. 6.5°C), but experience 2–4% reduced survival (Arnberg et al., 2013).

Salmosan® exposure affected survival in prawns, with post-molt prawns demonstrating higher sensitivity (lower LC50 values) to Salmosan® compared to intermolt prawns that experienced no mortality at any concentration tested. Differential sensitivity in crustaceans at various molt stages has been described in several studies for other environmental contaminants. For example, *Daphnia magna* that molted during exposure or just before exposure were more sensitive to chromate than those who did not molt (Lee & Buikema, 1979). Post-molt *Crangon crangon* shrimp were more sensitive to copper and zinc (but not cadmium) compared to intermolt shrimp (Price & Uglow, 1979). Burridge et al. (2005) describe seasonal fluctuations in the sensitivity of American lobsters to emamectin benzoate, with the

most sensitive stage being lobsters that had recently molted. Bechmann et al. (2018) found that *P. borealis* shrimp that molted during exposure to diflubenzuron experienced higher rates of mortality than those that did not molt.

The 3 × 1-hr LC50 values estimated for post-molt prawns ranged from 17 to 40 μg/L. These values are comparable to those for the American lobster that were reported as 1-hr LC50 values between 20.70–37.70 μg/L for Stage II larvae and 24.8 μg/L for adults (Burridge, Lyons, Wong, MacKeigan, & Geest, 2014; Pahl & Opitz, 1999). Conversely, American lobster in the Stage I larval phase was more resilient with a reported 1-hr LC50 value >85.5 μg/L (Burridge et al., 2014). While the adult intermolt American lobster is more sensitive than intermolt adult Pacific spot prawn, the overall life stage sensitivity spans a similar range for both species. Stage I and Stage II larvae of the European lobster (*Homarus gammarus*) 1-hr LC50 values ranged from 24 to 76 μg/L, respectively (Parsons, Escobar-Lux, Saevik, Samuelsen, & Agnalt, 2020). In Atlantic Canada, this range of life stage sensitivity to the American lobster led to restrictions on the use of Salmosan® near American lobster rearing facilities (PMRA, 2016). Other crustacean species for which reported 1-hr LC50 values are available showed less sensitivity compared to the post-molt prawns in this study and include the *Mysid sp*. with a reported 1-hr LC50 value >85.5 μg/L, and the sand shrimp with a reported 1-hr LC50 value of >85.5 μg/L (Burridge et al., 2014).

There are several possible reasons that Salmosan® exposures are more lethal to post-molt prawns compared to intermolt prawns. These reasons can generally be categorized as an increased uptake (and higher internal dose) or an increased sensitivity to the compound. The internal dose of azamethiphos is most likely increased in post-molt prawns through an increase in uptake by the continuous intake of water immediately before and following ecdysis (Hosamani et al., 2017). Following ecdysis, crustaceans take in seawater to increase their body volume, which applies pressure against their new, malleable exoskeleton and promotes growth while the exoskeleton hardens (Chang, 1995; Hosamani et al., 2017). Therefore, post-molt prawns in this study were likely subjected to a higher internal dose than the intermolt prawns. Increased internal dose was speculated to be the primary contributor to increased toxicant susceptibility in the post-molt stage in lobsters as well (Burridge et al., 2005; Lee & Buikema, 1979; Price & Uglow, 1979).

Ecdysis in crustaceans involves a complex interplay of endocrine and neuroendocrine factors that mediate significant alterations in tissue metabolism and energy storage, hemolymph composition and osmoregulation, muscle biochemistry, respiration and circulation, functional and structural alterations in the epidermis and exoskeleton, and behavioral patterns (Chang, 1995). In addition to an increased internal dose, the sensitivity to environmental stressors (e.g., hypoxia, temperature) and anthropogenic chemicals such as azamethiphos can increase during the post-molt stage in decapod crustaceans (Hosamani et al., 2017). The mechanisms underlying this sensitivity increase can be varied, chemical-specific, or general. For example, the role of cholinergic signaling pathways in the control of many of the processes occurring during ecdysis is unknown, and the inhibition of the cholinergic system by azamethiphos may alter these some of these processes significantly. A molt-cycle-related decrease in metabolic rate and oxygen transport following ecdysis is a common trend among decapod crustaceans (Penkoff & Thurberg, 1982). In the blue swimmer crab (*Portunus pelagicus*), the transcription of enzymes related to cellular respiration decreases in the post-molt phase (Kuballa, Holton, Paterson, & Elizur, 2011). Hemocyanin levels have been shown to decrease in the post-molt stage of crustaceans (Kuballa et al., 2011). Further, post-molt decapod crustaceans allocate much of their available energy to the calcification of the new exoskeleton, as well as toward maintaining hemolymph isotonicity in the presence of increasing water volume (Price & Uglow, 1979). Therefore, the post-molt prawns in this study may have had less energy available or ability to use available energy for azamethiphos biotransformation or compensation/repair of processes affected by neuronal inhibition compared to intermolt prawns.

Depressed chemosensory-mediated behaviors by Salmosan® exposure also occurred in prawns, suggesting sublethal neurological alterations that may be significant to prawn survival. In the 17°C treatment group, prawns exposed to Salmosan® at 50 and 100 μg/L azamethiphos exhibited a decreased response to L-glutamate compared to controls (Figure 2). Azamethiphos, as an organophosphate (OP), can desensitize post-synaptic muscle membranes to the neurotransmitter acetylcholine (ACh) at neuromuscular junctions, resulting in a general weakening or loss of muscular functioning (Colovic, Krstic, Lazarevic-Pasti, Bondzic, & Vasic, 2013) and reductions in behavioral

movements. ACh is also the primary neurotransmitter in chemosensory neuronal pathways in crustaceans (Florey, 1973), potentially leading to the depressed response to L-glutamate through an inability to detect the compound or diminish the stimulatory response to it. In either case, AChE inhibition caused by Salmosan® exposure may be the underlying mechanism leading to these measurable effects on the prawn nervous system.

Evidence was also seen for recovery from the effects of exposure to Salmosan®; a difference in the behavioral responses to L-glutamate between exposed and control prawns was seen at 24 but not at 96 hr following exposure (Figure 2). Previous studies have demonstrated that crustaceans, mollusks, and fish can recover from AChE inhibition following OP exposure. For example, European eel (*Anguilla anguilla*), European sea bass (*Dicentrarchus labrax*), and rainbow trout (*Oncorhynchus mykiss*) experienced reduced AChE activity levels in the brain after exposure to azamethiphos, which returned to baseline levels 4 to 7 days following exposure (Intorre et al., 2004). Atlantic salmon exposed to the OP fenitrothion experienced recovery from brain AChE inhibition within weeks; the recovery time depended on the extent of inhibition (Morgan, Fancey, & Kiceniuk, 1990). Penaeid shrimp (*Metapenaeus monoceros*) experienced some but not full recovery 7 days following AChE inhibition from the OPs phosphamidon and paramalathion exposure (Reddy & Rao, 1988). Recovery from AChE inhibition is likely driven by the de novo synthesis of AChE, as spontaneous enzyme reactivation (i.e., dephosphorylation of AChE) is slow and minor and would take longer to have any effect compared to de novo synthesis (Colovic et al., 2013; Intorre et al., 2004).

There was no evidence of an effect of Salmosan® exposure during the intermolt period on any of the molting endpoints measured (time to molt, molting success, CL growth during molt; $p > .05$; Figure 4). With respect to time to molt, previous studies have demonstrated that other neurotoxic agents (e.g., DDT and emamectin benzoate) induce molting (Waddy et al., 2002; Weis & Mantel, 1976). This is believed to be due to the interference of the release of molt inhibiting hormone (MIH) from the eyestalk, which is under control of the nervous system (Hosamani et al., 2017). In this study, there was no significant difference in the meantime to molt between the control groups and any treatment group. Since the major mechanism of action of azamethiphos is AChE inhibition, these results suggest that either the Salmosan® exposures in this study did not cause sufficient AChE inhibition to induce molting or that the cholinergic system does not play a large role in mediating prawn molting.

Across all treatment groups, there was a mean loss of mass in prawns over the course of the experiment that likely had implications for the CL growth measurement (Figure 4). The mean relative CL growth across all treatments ranged from 0.12 to 2.1%, whereas relative CL growth was expected to be close to 10% as reported in previous studies (Prentice & Rensel, 1977). Prawns were fed ad libitum three times a week and were monitored to ensure some fish feed pellets remained 1 hr post-feeding; suggesting that prawns fed until satiation or required longer feeding periods. Many factors may have reduced feeding and the assimilation of food. For example, prawns are nocturnal foragers, so they may not feed well under the experimental photoperiod (Butler, 1964). Molting has been shown to result in a decrease in mass and length in Antarctic krill (*Euphausia superba*) when food is in short supply (Ikeda & Dixon, 1982). Future studies that use growth during molting as an endpoint should be aware of selecting an appropriate diet and growth should be monitored continuously.

Results from the acute lethality and behavioral tests show that temperature exacerbates Salmosan® toxicity. Post-molt prawns were more sensitive at higher temperatures, and a depressed response to L-glutamate was seen in prawns exposed to Salmosan® at the high temperature. A similar association between azamethiphos toxicity and temperature has been demonstrated in American lobsters which experienced significantly greater azamethiphos-induced mortality at 12°C compared to 10°C (Pahl & Opitz, 1999). Additionally, two complementary studies by Burridge et al. (2005, 2008) reported seasonal variation in the sensitivity of American lobsters to azamethiphos, with a greater impact on mortality and spawning incidence occurring at higher water temperatures.

One major factor that may contribute to increased toxicity is increased metabolic rate and oxygen demand with increasing ambient temperature (González et al., 2010; Manush et al., 2004), demonstrated in crustaceans (e.g., adult white shrimp [*Penaeus vanname*] and giant freshwater prawn [*Macrobrachium rosenergii*]), even after temperature-acclimation periods of up to 30 days (González et al., 2010; Manush et al., 2004). In response to increased oxygen demand, ventilation rates increase, increasing chemical uptake and the resulting internal dose (Heugens et al., 2003).

This has been demonstrated in *D. magna* that had higher internal concentrations of cadmium following exposures at higher acclimation temperatures compared to cooler temperatures (Heugens et al., 2003). It should be noted that the effect of temperature on metabolic rate and oxygen uptake is more pronounced following acute temperature changes compared to long-term temperature acclimation (Kennedy, Gill, & Walsh, 1989).

The temperature-dependence of toxicity in ectotherms is well known; however, the directional change of toxicity is chemical-dependent (Kennedy & Walsh, 1997). Due to the variety and complexity of cellular and molecular processes influenced by temperature, the effects of temperature on chemical sensitivity are difficult to predict. For example, temperature can affect xenobiotic biotransformation rates, antioxidant responses, and the transcription of heat shock proteins (Kennedy & Walsh, 1997; Portner & Knust, 2007). The relationship between temperature and chemical sensitivity depends on the duration of acclimation to a new temperature (Kennedy et al., 1989), and temperature-dependent toxicity most often follows one of two models: (1) increasing toxicity with temperature or (2) increasing toxicity in either direction away from an optimal temperature (Zhou, Wang, Lau, Xu, & Leung, 2014). In invertebrates, the first model is most common and is likely due to the ability to enter protective states of dormancy at decreased temperatures (Portner & Knust, 2007). The results of the present study agree with this generalization, demonstrating increasing Salmosan® toxicity with increasing exposure temperature.

The data generated have implications to regulatory aspects of chemotherapeutant use in the aquaculture industry. Sublethal effects occurred in intermolt prawns at concentrations of 50–100 μg/L and with generated LC50 values in post-molt prawns between 17 and 40 μg/L. Target concentration within net pens in standard use is 100 μg/L azamethiphos; however, proximal organisms will be exposed to short-term, low concentrations of azamethiphos due to immediate dilution in the water column following its release. The dilution of azamethiphos following release is variable and depends on factors including tidal amplitude, currents, water depth and weather. In a field study, reported maximum azamethiphos concentrations within 10 m of a fish farm were 10 μg/L following the release of Salmosan® (Ernst et al., 2014). Burridge et al. (2000) used estimated concentrations surrounding net pens following release and determined that a 10^3 dilution factor should be used for concentration predictions 10 m from the net pen 3 hr post-release, and based on a target concentration of 100 μg/L (azamethiphos), the range of concentrations is predicted to be 0.1 to 10 μg/L.

This study reports results from repeated (3×) short-term (1-hr) exposures. Repeated exposures are less common in the literature for the determination of LC50 values and other toxicological parameters, and therefore difficult to compare to other chemicals and species. Organisms may be subjected to repeated pulses of Salmosan® as farm managers may apply consecutive treatments within a site to target all life stages of the sea louse, or to treat multiple pens within one site (Burridge et al., 2008; Dounia et al., 2016). Further, proximal farms may be treating outbreaks simultaneously, as outbreaks can spread between sites. These multiple exposures would likely occur within 6 hr and/or repeated at intervals of approximately 12 hr, to coincide with an outgoing tide as stated by the label protocol (PMRA, 2016).

Toxicity data on anti-sea lice chemotherapeutants are required to select formulations and application protocols that are effective in controlling sea lice outbreaks while estimating risks and then minimizing non-target organism effects. Results here suggest that the risk of Salmosan® released to spot prawns is low for intermolt prawns, as the only effects were observed under concentrations higher than found following application. Environmentally relevant concentrations are estimated to be up to 10 μg/L after dilution; therefore, the potential for mortality to occur in post-molt prawns following the release of Salmosan® from net pens exists (Burridge et al., 2000; Ernst et al., 2014). The risk to the overall population of prawns near a net pen will depend on the proportion of prawns in the post-molt phase, water temperature, and factors influencing dilution (Ernst et al., 2014). Sea lice outbreaks tend to occur during returning salmon migrations and warmer water temperatures in August and September, coinciding with a peak molting season for juvenile and adult prawns (Costello, 2006). Therefore, there is the potential for Salmosan® treatments to occur when prawns are most sensitive and under higher water temperatures. Adult prawns do not migrate to a significant extent and are estimated to remain within a 10 km home range throughout their entire life (Bower, Meyer, & Boutillier, 1996); they are at risk of effects in net pen areas following repeated exposure.

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